PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A61K 38/08, 38/17, 38/22 | A1

(11) International Publication Number: **WO 00/27419**

(43) International Publication Date: 18 May 2000 (18.05.00)

(21) International Application Number: PCT/US99/26237

(22) International Filing Date: 8 November 1999 (08.11.99)

(30) Priority Data:
P 98 01 05608 6 November 1998 (06.11.98) AR
P 99 01 00677 23 February 1999 (23.02.99) AR

(71) Applicant *(for all designated States except US)*: STERRENBELD BIOTECHNOLOGIE NORTH AMERICA, INC. [US/US]; 1209 Orange Street, Wilmington, DE 19801 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: CARCAGNO, Carlos, Miguel [AR/AR]; Guevara 275, 1427 Buenos Aires (AR). CRISCUOLO, Marcelo [AR/AR]; Guevara 349, 1427 Capital Federal (AR). MELO, Carlos [AR/AR]; Alsina 2737, Del Viso, Pcia, 1669 Buenos Aires (AR). VIDAL, Juan, Alejandro [AR/AR]; Guise 1760, 9th floor, Apartment A, 1425 Buenos Aires (AR).

(74) Agents: GOLDSTEIN, Jorge, A. et al.; Sterne, Kessler, Goldstein & Fox P.L.L.C., Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: METHOD FOR OBTAINING LYOPHILIZED PHARMACEUTICAL COMPOSITIONS OF RECOMBINANT HUMAN ERYTHROPOIETIN STABLE AT ROOM TEMPERATURE

(57) Abstract

The present invention relates, in general, to a lyophilized pharmaceutical composition comprising recombinant human erythropoietin, which retains at least 95 % of its biological activity after 24 months at room temperature. The present invention also relates to a method for producing a recombinant human erythropoietin compound, which is stable at room temperature.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

Method for Obtaining Lyophilized Pharmaceutical Compositions of Recombinant Human Erythrop ietin Stable at Room Temperature

Background of the Invention

Field of the Invention

The present invention relates, in general, to a method for obtaining lyophilized pharmaceutical compositions of recombinant human Erythropoietin (EPO) stable at room temperature. The present invention also refers to the EPO formulations thus obtained.

Background Information

EPO is a glycoprotein that stimulates erythroblast differentiation in the bone marrow, thus increasing the circulating blood erythrocyte count. The mean life of erythrocytes in humans is 120 days and therefore, a human being losses 1/120 erythrocytes each day. This loss must be continuously restored to maintain a stable level of red blood cells.

The existence of EPO was first postulated by the turn of the century and was definitely proved by Reissman and Erslev early in the '50s. See Carnot, et al., *C.R. Acad. Sci.* (France), 143, 384-6 (1906); Carnot, et al., *C.R. Acad. Sci.* (France), 143, 432-5 (1906); Carnot, et al., *C.R. Soc. Biol.*, 111, 344-6 (1906); Carnot, *C.R. Soc. Biol.*, 111, 463-5 (1906); Reissman, *Blood*, 1950, 5, 372-80 (1950) and Erslev, *Blood*, 8, 349-57 (1953). Reissman and Erslev's experiments were promptly confirmed by other researchers. See Hodgson, et al., *Blood*, 9, 299-309 (1954); Gordon, et al., *Proc. Soc. Exp. Biol. Med.*, 86, 255-8 (1954) and Borsook, et al., *Blood*, 9, 734-42 (1954).

The identification of the EPO production site in the organism was an issue of debate. Successive experiments led to identify the kidney as the main organ and peritubular interstitial cells as the synthesis site. See Jacobson, et al., *Nature*, 179, 633-4 (1957); Kuratowska, et al., *Blood*, 18, 527-34 (1961); Fisher, *Acta*

Hematol., 26, 224-32 (1961); Fisher, et al., *Nature*, 205, 611-2 (1965); Frenkel, et al., *Ann. N.Y. Acad. Sci.*, 149, 1, 292-3 (1968); Busuttil, et al., *Proc. Soc. Exp. Biol. Med.*, 137, 1, 327-30 (1971); Busuttil, *Acta Haematol.*, (Suiza), 47, 4, 238-42 (1972); Erslev, *Blood*, 44, 1, 77-85 (1974); Kazal, *Ann. Clin. Lab. Sci.*, 5, 2, 98-109 (1975); Sherwood, et al., *Endocrinology*, 99, 2, 504-10 (1976); Fisher, *Ann. Rev. Pharmacol. Toxicol.*, 28, 101-22 (1988); Jelkmann, et al., *Exp. Hematol.*, 11, 7, 581-8 (1983); Kurtz, et al., *Proc. Natl. Acad. Sci.*, (EE.UU.), 80, 13, 4008-11 (1983); Caro, et al., *J. Lab. Clin. Med.*, 103, 6, 922-31 (1984); Caro, et al., *Exp. Hematol.*, 12, 357 (1984); Schuster, et al., *Blood*, 70, 1, 316-8 (1986); Bondurant, et al., *Mol. Cell. Biol.*, 6, 7, 2731-3 (1986); Bondurant, et al., *Mol. Cell. Biol.*, 6, 7, 2731-3 (1986); Schuster, et al., *Blood*, 71, 2, 524-7 (1988); Koury, et al., *Blood*, 71, 2, 524-7 (1988); Lacombe, et al., *J. Clin. Invest.*, 81, 2, 620-3 (1988); Koury, et al., *Blood*, 74, 2, 645-51 (1989).

A smaller proportion, ranging from 10% to 15% of total EPO, is produced by the liver in adults. See Naughton, et al., *J. Surg. Oncol.*, 12, 3, 227-42 (1979); Liu, et al., *J. Surg. Oncol.*, 15, 2, 121-32 (1980); Dornfest, et al., *Ann. Clin. Lab. Sci.*, 11, 1, 37-46 (1981); Dinkelaar, et al., *Exp. Hematol.*, 9, 7, 796-803 (1981); Caro, et al., *Am. J. Physiol.*, 244, 5 (1983); Dornfest, et al., *J. Lab. Clin. Med.*, 102, 2, 274-85 (1983); Naughton, et al., *Ann. Clin. Lab. Sci.*, 13, 5, 432-8 (1983); Jacobs, et al., *Nature*, 313, 6005, 806-10 (1985); Erslev, et al., *Med. Oncol. Tumor. Pharmacother.*, 3, 3-4, 159-64 (1986). The EPO produced is directly proportional to the extent of tissular hypoxia and its expression rises by increasing the number of the EPO producing cells.

EPO has shown great efficiency in the treatment of anemia, especially anemia derived from renal failure. See Eschbach, et al., *N. England J. of Med.*, 316, 2, 73-78 (1987); Krane, *Henry Ford Hosp. Med. J.*, 31, 3, 177-181 (1983). Its therapeutical usefulness, however, has been limited due to the unavailability of a massive production method. The quantity and quality of the EPO obtained by the extractive systems known were insufficient. Recently, the use of recombinant DNA technology has made it possible to obtain large amounts of proteins. The application of these techniques to eukaryotic cells has allowed a

large-scale production of EPO. See patents US 5,688,679 (to Powell), US 5,547,933 (to Lin), US 5,756,349 (to Lin), US 4,703,008 (to Lin) and US 4,677,195 (to Hewick et al.)

The present EPO pharmaceutical formulations are unstable at room temperature and require uninterrupted storage under low temperatures. The EPO pharmaceutical formulations now under use may become inactivated and result in degraded EPO when exposed to room temperature. This circumstance may render the present EPO formulations antigenic and toxic for human utilization specially in developing countries, where high temperatures and an inadequate infrastructure often prevent the preservation of pharmaceutical products under continuous refrigeration. The application of inactivated or degraded EPO to human beings will not produce the expected beneficial results.

At the present, it is fundamental for therapeutical safety reasons to devise stable EPO formulations at room temperature. The formulations obtained by the procedure described in the claimed invention addresses this need.

Summary of the Invention

One advantage of the present invention is the obtention of stable EPO formulations at room temperature adequate for use in human beings. The EPO pharmaceutical formulations obtained by the claimed invention retain more than 95 % of their biological activity after a 24-month period at room temperature. Another advantage of the pharmaceutical forms obtained by the claimed invention is their stability in comparison to the existent liquid EPO formulations. The liquid EPO solutions are easily denatured due to temperature conditions and shaking during transportation and handling. This phenomenon is avoided with the EPO pharmaceutical formulations obtained by the claimed invention which render EPO formulations more resistant to shaking and changes of temperature.

The unexpected benefits of the EPO pharmaceutical formulations obtained by the claimed invention are achieved through the combined effect of the excipients and the lyophilization process utilized.

The claimed pharmaceutical formulations are obtained by first preparing galenic formulations containing EPO. The galenic formulations are allotted in vials, which are then lyophilized in a pre-cooled chamber. The lyophilization chamber pressure is reduced thereafter and its temperature increased at a constant rate. The system is maintained under constant pressure and temperature until the EPO formulations are dry. The vials containing the EPO lyophilized pharmaceutical forms are then hermetically sealed to preserve a low humidity level and sterility. All these processes are performed under strict sterile conditions.

Detailed Description of the Invention

In one embodiment, the present invention relates to a lyophilized pharmaceutical composition comprising EPO, which retains more than 95% of its activity after 24 months at room temperature.

The EPO is preferably obtained through culturing recombinant mammalian cells, followed by purification. Preferably, the recombinant human EPO expressing cells are selected from the group comprising mammalian cells. Preferably said mammalian cells are selected from the group comprising CHO, COS, BHK, Namalwa, and HeLa, and even more preferably said mammalian cells are CHO cells.

Preferred recombinant mammalian cells comprise a vector which comprises a nucleotide sequence encoding the EPO polypeptide, a viral promoter and a viral terminator. Preferred host cell vectors confer resistance to both methotrexate and neomycin-derived antibiotics. Preferably, the EPO nucleic acid molecule comprises the nucleic acid molecule described in Lin, "DNA Sequences Encoding Erythropoietins," U.S. Patent No. 4,703,008. Preferably, the viral promoter is a SV40 early promoter.

The EPO protein can be further purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose

chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. A preferred method of further purifying the EPO comprises treating cell culture supernatants comprising EPO by a combination of the following steps: (a) differential precipitation, (b) hydrophobic interaction chromatography, (c) diafiltration, (d) anionic exchange chromatography, (e) cationic exchange chromatography and (f) molecular exclusion chromatography. Preferably, said steps are performed in the following order: (a), (b), (c), (d), (e), and (f).

The invention further provides a lyophilized pharmaceutical composition comprising EPO, which retains more than 95% of its activity after 24 months at room temperature and comprises sugar, salts and human albumin. Preferably, said sugar comprises mannitol. Preferably, said salts comprise NaCl, NaH_2PO_4 and $Na_2HPO_4 \cdot 12H_2O$.

In another embodiment, the present invention relates to a lyophilized pharmaceutical composition comprising EPO which retains more than 95% of its activity after 24 months at room temperature wherein mannitol is present in amounts ranging from about 10.0 to about 50.0 mg per vial.

The present invention further relates to a lyophilized pharmaceutical composition comprising EPO which retains more than 95% of its activity after 24 months at room temperature wherein NaCl is present in amounts from 2.0 to about 10.0 mg per vial.

In another embodiment, the present invention provides a lyophilized pharmaceutical composition comprising EPO, which retains more than 95% of its activity after 24 months at room temperature wherein NaH_2PO_4 is present in amounts ranging from 0.5 to 3.0 mg per vial.

In a further embodiment, the present invention relates to a lyophilized pharmaceutical composition comprising EPO which retains more than 95% of its activity after 24 months at room temperature wherein $Na_2HPO_4\_12H_2O$ is present in amounts ranging from 2.0 to about 4.0 mg per vial.

In a further embodiment, the present invention relates to a lyophilized pharmaceutical composition comprising EPO which retains more than 95 % of

its activity after 24 months at room temperature wherein human albumin is present in amounts ranging from about 1.0 mg to about 10.0 mg per vial.

In another embodiment, the present invention relates to a lyophilized pharmaceutical composition comprising EPO which retains more than 95% of its activity after 24 months at room temperature wherein recombinant human EPO is present in amounts ranging from about 500 to about 20,000 IU.

The invention further provides a method for producing an EPO compound wherein said compound is stable at room temperature, comprising loading a first EPO compound into a container, wherein said container is at a temperature equal or inferior to -30°C. Preferably, said temperature is -30°C, -35°C or -40°C.

The invention further provides a method for producing an EPO compound wherein said compound is stable at room temperature, comprising incubating a first EPO compound at a temperature equal or inferior to -30°C under atmospheric pressure for 4 hours or for a longer period. Preferably said period is of more than 5 hours, but less than 10 hours.

In another embodiment, the invention further provides a method for producing an EPO compound wherein said compound is stable at room temperature, comprising incubating EPO at a pressure equal or inferior to 30 absolute microns for 1 hour or for a longer period. Preferably said pressure is of about 30 absolute microns. Preferably said time is 2 hours, and even more preferably said time is 12 hours.

The invention further provides a method for producing an EPO compound wherein said compound is stable at room temperature, comprising raising the temperature of a lyophilizer containing an EPO compound at a rate equal or inferior to 3°C per hour until reaching at least 25°C, while keeping pressure values equal or inferior to 5 absolute microns. Preferably, said temperature is increased by 1°C per hour and even more preferably, said temperature is increased by 3°C per hour. Preferably said temperature is increased to about 30° C.

The invention further relates to a method for producing an EPO compound wherein said compound is stable at room temperature, and contained in vials, which are hermetically sealed.

The present invention is described in further detail in the following non-limiting examples.

Examples

Example 1 Lyophilization Procedure A

Under strict sterile conditions, EPO aliquots in 1 ml vials were made with each of the four galenic formulations described in Table 1. For each galenic formulation a total 1,000 vials were prepared. A FIC 150 lyophilizer was pre-cooled at a temperature below -30°C. The vials were then loaded into the lyophilizer.

Subsequently, the vials were frozen at -40°C for 10 hours at atmospheric pressure. The pressure was then lowered to 30 absolute microns and the vials were stored for an additional 12-hour period at -40°C.

Thereafter, the temperature was increased at a rate of 2 °C to 3 °C per hour until a final temperature of 30°C was reached. A second drying step was performed by decreasing the lyophilizer pressure to a range between 1 to 5 absolute microns for 12 additional hours at 30°C.

Finally, the vials containing the lyophilized product were capped and sealed in a sterile area under laminar flow.

Example 2 Lyophilization Procedure B

Under strict sterile conditions, EPO aliquots were dispensed in 1 ml vials with each of the four galenic formulations described according to Table 1. For each galenic formulation a total 1,000 vials were prepared. A FIC 150 lyophilizer

was pre-cooled at a temperature below -30°C. The vials were then loaded into the lyophilizer.

Subsequently, the vials were frozen at -40°C for 6 hours at atmospheric pressure. The pressure was then lowered to 30 absolute microns and the vials were stored for an additional 2 hours at -40°C.

Thereafter, the temperature was increased at a rate of 1°C to 2°C per hour until a final temperature of 30°C was reached. A second drying step was performed by decreasing the lyophilizer pressure to a range between 1 to 5 absolute microns for 12 additional hours at 30°C.

Finally, the vials containing the lyophilized product were capped and sealed in a sterile area under laminar flow.

Example 3 Stability Tests

A sample of the vials containing each of the four EPO formulations, prepared and lyophilized according to the described method, was submitted to the following quality control tests:

A Western blot assay was performed to verify the absence of degraded or aggregated material.

The EPO formulations were assayed with an ex-hypoxic polycythemic mice test to determine *in vivo* biological activity.

Protein mass was determined by radioimmunoassay;

The formulation specific activity was determined as the ratio of the values obtained for assays 2 and 3.

Once the quality of the pharmaceutical forms obtained was duly tested, the remaining vials were preserved at room (30°C) temperature for 24 months. For comparison, vials containing the lyophilized product were preserved at cold (2-8°C) temperature for 24 months.

Quality control assays were periodically repeated. After 24 months of storage at room and cold temperature, the EPO preparations obtained according

to the claimed invention showed to preserve at least 95 % of its initial biological activity. See Tables 2 and 3.

Table 1. Galenic Formulations

r-hu-EPO 1000 IU Formulation	
Mannitol (mg)	25.0
NaCl (mg)	3.2
NaH_2PO_4 (mg)	1.4
$Na2HPO_4.12H_20$ (mg)	4.0
H. Albumin (mg)	2.5
R-hu-EPO (IU)	1000
H_20 c.s.p. (ml)	1
r-hu-EPO 2000 IU Formulation	
Mannitol (mg)	50.0
NaCl (mg)	6.4
NaH_2PO_4 (mg)	2.8
$Na_2HPO_4.12H_20$ (mg)	8.0
H. Albumin H.(mg)	5.0
R-hu-EPO (IU)	2000
H_20 c.s.p. (1 ml)	1
r-hu-EPO 4000 IU Formulation	
Mannitol (mg)	50.0
NaCl (mg)	6.4
NaH_2PO_4 (mg)	2.8
$Na_2HPO_4.12H_20$ (mg)	8.0
H. Albumin (mg)	5.0
R-hu-EPO (I.U.)	4000
H_20 c.s.p. (ml)	1

r-hu-EPO 10000 IU Formulation

Mannitol (mg)	25.0
NaCl (mg)	3.2
NaH_2PO_4 (mg)	1.4
$Na_2HPO4.12H_20$ (mg)	4.0
H. Albumin (mg)	2.5
R-hu-EPO (IU)	10000
H_20 c.s.p. (ml)	1

Table 2. Lyophilized Product Stability at 2-8 °C

A. r-Hu-EPO 1000 IU Injectable Lyophilized Formulation

LOT: BS-1051/E
CONTENT: 1,000 IU/vial
TEMP: 2-8°C

Time months	Lyoph. Product		Reconstituted Product[1]					
	Aspect	Solubility	Physchem Analyses[2]	Microbiological [3]	Endo-Toxins[4]	Biological Activity Iu/vial[5]	Biological Activity % Label	Degradation Products[6]
0	S	I	S	STERILE	<10	1050	105.0	_1 %
3	S	I	S	---	---	---	---	_1 %
6	S	I	S	STERILE	<10	1055	105.5	_1 %
9	S	I	S	---	---	---	---	_1 %
12	S	I	S	STERILE	<10	1060	106.0	_1 %
18	S	I	S	STERILE	<10	1030	103.0	_1 %
24	S	I	S	STERILE	<10	1015	101.5	_1 %

B. r-Hu-EPO 2000 Injectable Lyophilized Formulation

LOT: BS-2051/E
CONTENT: 2,000 IU/vial
TEMP: 2-8°C

Time Months	Lyoph. Product		Reconstituted Product[1]					
	Aspect	Solubility	Physchem Analyses[2]	Microbiological [3]	Endo-Toxins[4]	Biological Activity Iu/vial[5]	Biological Activity % Label	Degradation Products[6]
0	S	I	S	STERILE	<10	2020	101.0	_1 %
3	S	I	S	---	---	---	---	_1 %
6	S	I	S	STERILE	<10	2010	100.5	_1 %
9	S	I	S	---	---	---	---	_1 %
12	S	I	S	STERILE	<10	2030	101.5	_1 %
18	S	I	S	STERILE	<10	1990	99.5	_1 %
24	S	I	S	STERILE	<10	1950	97.5	_1 %

C. r-Hu-EPO 4000 Injectable Lyophilized Formulation

LOT: BS-4051/E
CONTENT: 4,000 IU/vial
TEMP: 2-8 °C

Time Months	Lyoph. Product		Reconstituted Product[1]					
	Aspect	Solubility	Physchem Analyses[2]	Microbiological[3]	Endo-Toxins[4]	Biological Activity Iu/vial[5]	Biological Activity % Label	Degradation Products[6]
0	S	I	S	STERILE	<10	4100	102.5	_ 1 %
3	S	I	S	---	---	---	---	_ 1 %
6	S	I	S	STERILE	<10	4080	102.0	_ 1 %
9	S	I	S	---	---	---	---	_ 1 %
12	S	I	S	STERILE	<10	4110 .	102.7	_ 1 %
18	S	I	S	STERILE	<10	4050	101.2	_ 1 %
24	S	I	S	STERILE	<10	3970	99.2	_ 1 %

D. r-Hu-EPO 10000 Injectable Lyophilized Formulation

LOT: BS-10061/E
CONTENT: 10,000 IU/vial
TEMP: 2-8 °C

Time Months	Lyoph. Product		Reconstituted Product[1]					
	Aspect	Solubility	Physchem Analyses[2]	Microbiological[3]	Endo-Toxins[4]	Biological Activity Iu/vial[5]	Biological Activity % Label	Degradation Products[6]
0	S	I	S	STERILE	<10	10450	104.5	_ 1 %
3	S	I	S	---	---	---	---	_ 1 %
6	S	I	S	STERILE	<10	10350	103.5	_ 1 %
9	S	I	S	---	---	---	---	_ 1 %
12	S	I	S	STERILE	<10	10400	104.0	_ 1 %
18	S	I	S	STERILE	<10	10300	103.0	_ 1 %
24	S	I	S	STERILE	<10	10100	101.0	_ 1 %

S: Satisfactory
I: Immediate

(1) The 1,000 UI and 10,000 UI ampoules were reconstituted in 1.0 ml of pyrogen free sterile water. The 2,000 UI and 4,000 UI ampoules were reconstituted in 2.0 ml of pyrogen free sterile water.

(2) Physicochemical analyses include aspect - color - pH - conductivity – osmolarity.

(3) Microbiological control according to USP XXII - Chapter 71: 1483.

(4) LAL-TEST according to USP XXII Chapter 85: 1493.

(5) Biological activity measured *in vivo* in the ex-hypoxic polycythemic mice assay.

(6) Degradation products, according to Western blot technique.

The stability of all product formulations utilizing the claimed process was evaluated satisfactory after a 24-month storage period at 2-8°C.

Table 3. Lyophilized Product Stability at 30 °C

A. *r-Hu-EPO 1000 IU Lyophilized Injectable Formulation*

LOT: E-01-2105-F
CONTENT: 1,000 IU/vial
TEMP: 30°C

Time Months	Lyoph. Product		Reconstituted Product[1]					
	Aspect	Solubility	Physchem Analyses[2]	Microbiological[3]	Endo-Toxins[4]	Biological Activity Iu/vial[5]	Biolo-gical Activity % Label	Degradation Products[6]
0	S	I	S	STERILE	<10	1050	105.0	_1 %
3	---	---	---	---	---	---	---	---
6	S	I	S	STERILE	<10	1030	103.0	_1 %
9	---	---	---	---	---	---	---	---
12	S	I	S	STERILE	<10	1040	104.0	_1 %

18	---	---	---	---	---	---	---	---
24	S	I	S	STERILE	<10	1015	101.5	_ 1 %

B. *r-Hu-EPO 2000 Injectable Lyophilized Formulation*

LOT: E-02-2105-F
CONTENT: 2,000 IU/vial
TEMP: 30°C

Time Months	Lyoph. Product		Reconstituted Product[1]					
	Aspect	Solubility	Physchem Analyses[2]	Microbiological[3]	Endo-Toxins[4]	Biological Activity Iu/vial[5]	Biological Activity % Label	Degradation Products[6]
0	S	I	S	STERILE	<10	2150	107.5	_ 1 %
3	---	---	---	---	---	---	---	---
6	S	I	S	STERILE	<10	2080	104.0	_ 1 %
9	---	---	---	---	---	---	---	---
12	S	I	S	STERILE	<10	2040	102.0	_ 1 %
18	---	---	---	---	---	---	---	---
24	S	I	S	STERILE	<10	2060	103.0	_ 1 %

C. *r-Hu-EPO 4000 Injectable Lyophilized Formulation*

LOT: E-04-2105-F
CONTENT: 4,000 IU/vial
TEMP: 30°C

Time Months	Lyoph. Product		Reconstituted Product[1]					
	Aspect	Solubility	Physchem Analyses[2]	Microbiological[3]	Endo-Toxins[4]	Biological Activity Iu/vial[5]	Biological Activity % Label	Degradation Products[6]
0	S	I	S	STERILE	<10	4200	105.0	_ 1 %
3	---	---	---	---	---	---	---	---
6	S	I	S	STERILE	<10	4140	103.5	_ 1 %
9	---	---	---	---	---	---	---	---
12	S	I	S	STERILE	<10	4020	100.5	_ 1 %
18	---	---	---	---	---	---	---	---

24	S	I	S	STERILE	<10	4050	101.3	_ 1 %

D. *r-Hu-EPO 10000 Lyophilized Injectable Formulation*

LOT: E-10-2105-F
CONTENT: 10,000 IU/vial
TEMP: 30°C

Time Months	Lyoph. Product		Reconstituted Product(1)					
	Aspect	Solubility	Physchem Analyses(2)	Microbiological(3)	Endo-Toxins (4)	Biological Activity Iu/vial(5)	Biological Activity % Label	Degradation Products(6)
0	S	I	S	STERILE	<10	10800	108.0	_ 1 %
3	---	---	---	---	---	---	---	---
6	S	I	S	STERILE	<10	10200	102.0	_ 1 %
9	---	---	---	---	---	---	---	---
12	S	I	S	STERILE	<10	10040	100.4	_ 1 %
18	---	---	---	---	---	---	---	---
24	S	I	S	STERILE	<10	10500	105.0	_ 1 %

S: Satisfactory
I: Immediate

(1) The 1,000 UI and 10,000 UI ampoules were reconstituted in 1.0 ml of pyrogen free sterile water. The 2,000 UI and 4,000 UI ampoules were reconstituted in 2.0 ml of pyrogen free sterile water.

(2) Physicochemical analyses include aspect - color - pH - conductivity – osmolarity.

(3) Microbiological control according to USP XXIII Chapter 71.

(4) LAL-TEST according to USP XXIII Chapter 85.

(5) Biological activity measured *in vivo* in the ex-hypoxic polycythemic mice assay.

(6) Degradation products, according to Western blot technique.

The stability of all product formulations utilizing the claimed process was evaluated satisfactory after a 24 months storage period at 30°C.

* * * * *

All publications mentioned hereinabove are hereby incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

What Is Claimed Is:

1. A lyophilized pharmaceutical composition comprising recombinant human erythropoietin which retains at least 95 % of its biological activity after 24 months at room temperature.

2. The pharmaceutical composition of claim 1, comprising: sugar, salts and human albumin.

3. The pharmaceutical composition of claim 2, wherein said sugar comprises mannitol.

4. The pharmaceutical composition of claim 2, wherein said salts are selected from the group comprising: NaCl, NaH_2PO_4 and $Na_2HPO_4 \cdot 12H_2O$.

5. The pharmaceutical composition of claim 2 comprising: NaCl, NaH_2PO_4 and $Na_2HPO_4 \cdot 12H_2O$.

6. The composition of claim 1, comprising:

Mannitol (mg)	10.0-50.0
NaCl (mg)	2.0-10.0
NaH_2PO_4 (mg)	0.5-3.0
$Na_2HPO_4 \cdot 12H_2O$ (mg)	2.0-4.0
Human Albumin (mg)	1.0-10.0
R-hu-EPO (IU)	500-20,000

7. A method for producing a recombinant human erythropoietin compound wherein said compound is stable at room temperature and suitable for use in humans, comprising:

loading a first EPO compound into a container, wherein said container is at a temperature equal to or below -30 °C, under sterile conditions;

freezing said first EPO compound at a temperature equal to or below -35°C under atmospheric pressure for 5 hours or more;

reducing the pressure at the lyophilizer till 30 absolute microns or less for two hours or more; and

raising the temperature at a rate of 3°C or less per hour until reaching at least 25°C, while keeping pressure values equal to or below 5 absolute microns.

8. The method of claim 7, wherein said temperature of step (a) is selected from the group comprising: -30°C, -35°C and -40°C.

9. The method of claim 7, wherein said step (b) is performed for more than about 5 hours.

10. The method of claim 7, wherein said step (b) is performed for less than about 10 hours.

11. The method of claim 7, wherein said pressure in step (c) is about 30 absolute microns.

12. The method of claim 7, wherein said step (c) is performed for about 2 hours.

13. The method of claim 7, wherein said step (c) is performed for about 12 hours.

14. The method of claim 7, wherein said temperature of step (d) is increased by 1°C per hour, approximately.

15. The method of claim 7, wherein said temperature of step (d) is increased by 3°C per hour, approximately.

16. The method of claim 7, wherein said temperature of step (d) is increased tills about 30°C.

17. The method of claim 7, wherein said vials are hermetically sealed following said step (d).

18. The method of claim 7, wherein said vials are hermetically sealed in sterile area under laminar flow.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26237

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 38/08, 38/17, 38/22

US CL :514/8, 12, 21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/8, 12, 21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

West, US patent full, STN via medline, caplus, embase, search terms: proteins or polypeptides or peptide, stabliz? lyophilized, and excipient, salt, sugar, albumin, and erythropoietin.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,806,524 A (KAWAGUCHI et al.) 21 FEBRUARY 1989 (21.02.89), see entire document, especially column 1, line 30 through column 2, line 11, Examples 4, 6 and claims.	1-18
Y	WO 98/00530 A1 (MOLECULAR BIOLOGY RESOURCES, INC.) 08 JANUARY 1998 (08.01.98), see entire document, especially page 4, lines 20-30, page 6, lines 10-26, Example 4 on page 23 and page 24, lines 1-6.	1-18
Y	US 5,783,559 A (FLORIN-ROBERTSSON et al.)21 JULY 1998 (21.07.98), see entire document. especially column 4, lines 1-21, Examples 3 and 4 and claims.	1-18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
27 JANUARY 2000	18 FEB 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer FOZIA HAMUD Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★